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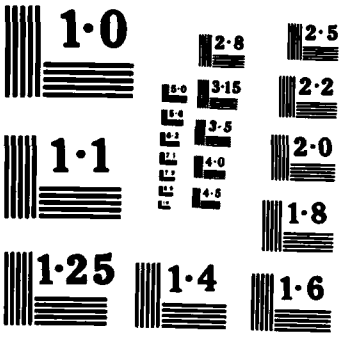

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TESTING EXPERIMENTAL COMPOUNDS AGAINST AMERICAN MUCOCUTANEOUS AND CUTANEOUS LEISHMANIASIS

FINAL REPORT

Jan S. Keithly, Ph.D.
July 1982
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Leishmania braziliensis panamensis	BALB/c mice	Ketoconazole DFMO
L. b. guyanensis	BCG	Pentostam Cream
L. mexicana mexicana	Bleomycin	Primaquine phosphate
L. m. amazonensis	Pentostam	8-aminoquinolines
L. donovani Khartoum	Glucantime	Imidazoles

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

Over a two year period under this contract, eight WRAIR compounds have been tested against two subspecies of L. mexicana, three species of Leishmania braziliensis, and L. donovani. Five of these were 8-aminoquinolines, two were imidazoles, and the last was primaquine phosphate. Systemic Pentostam was tested in combination with Pentostam cream and with BCG in each of these models, as were anti-trypanosome compounds alpha D,L-difluoromethylornithine (DFMO) + Bleomycin.

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In the visceral model, all aminoquinolines were as active as Pentostam, as shown by Pentostam Indices of 1.4 to 11.8. Spleens from these mice were all culture positive, however, and no cures were observed. None of the Therapeutic Indices for these drugs was competitive with Pentostam (TI = 1.20 to 2.00 versus 160), and three of the aminoquinolines tested were significantly toxic in mice. The LD50 for Pentostam in this model was 600 mkd, whereas that for the experimental compounds tested was 10 to 125 mkd. Cutaneous and mucocutaneous infections in BALB/c mice were only slightly altered by treatment with these drugs. Based upon these data, none of the aminoquinolines is recommended for further testing.

The imidazole ketoconazole and its acid hydrolysate are active against Trypanosoma and Leishmania species in vitro. Neither of these compounds was active against L. donovani infections in mice, probably because leishmania preferentially use serum cholesterol in vivo. Clinical trials reveal high doses for several months are necessary to promote healing. Neither the Pentostam nor Therapeutic Index of these imidazoles indicates further development would be useful. This is also the decision of its manufacturer.

Three types of combination chemotherapy were tested against visceral, cutaneous, and/or mucocutaneous infections in BALB/c mice. These included systemic and topical application of Pentostam against L. braziliensis, Pentostam and bacille Calmette-Guerin (BCG) against L. mexicana amazonensis, and DFMO in combination with Bleomycin against L. donovani infections.

Of the combinations tested, only 1% DFMO in the drinking water in combination with 3 mkd Bleomycin was competitive with Pentostam. Cures were achieved, and parasite burdens were suppressed whether this combination was given before, at time of, or after infection.

This is the first time in more than 30 years that a specific, non-toxic treatment against leishmaniasis is competitive with antimonials. The prophylactic effect of this combination, and the ease of delivery of DFMO suggest that this drug in combination with other known antileishmanial drugs, e.g., Pentostam, Pentamidine, and the best experimental ones, eg. Lampit, Radanil, WR 6026, should be given high priority. We recommend that experiments be designed to test DFMO with selected agents in each of our mouse test systems.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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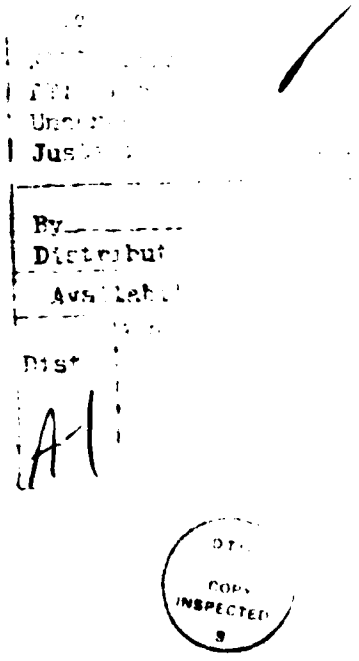

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SUMMARY OF RESEARCH

Contract DAMD17-80-C-0061 was initiated 1 July 1980 to serve as a Secondary Screening Program primarily to identify new antileishmanial drugs against New World cutaneous, mucocutaneous, and visceral leishmaniasis. Using 3 subspecies of the Leishmania braziliensis complex, two L. mexicana subspecies, and L. donovani Sudan strain 1S, we determined:

(1. the optimum conditions for rapid, reliable screening,
(2. the Effective Dose (ED) 50 and 90 for sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime) in these models,
(3. the efficacy of primaquine phosphate and five 8-aminoquinolines identified in the primary screening hamster model,
(4. the effect of combination chemotherapy using systemic Pentostam with its topical cream or with BCG, and
(5. the efficacy of two new antileishmanial drugs identified by rational approaches to drug therapy: ketoconazole and alpha D,L-difluoromethylornithine (DFMO).

BODY OF REPORT

I. Cutaneous and Mucocutaneous Test Systems

A. Protocol

Rapid, reliable cutaneous and mucocutaneous test systems were developed using Leishmania mexicana mexicana, L. m. amazonensis, L. braziliensis panamensis, and L. b. guyanensis infections in BALB/c mice. During the first year of this contract, promastigotes of these species were determined to be as effective as amastigotes in producing reliable lesions for screening, if cells were cultivated in complete Schneider's medium [CSM = Schneider's drosophila medium (GIBCO) + 15% v/v heat-inactivated fetal bovine serum (HyClone, Sterile Systems, Logan Utah)] to stationary phase [Annual Report 1, Figures 1, 2, 4, & 5 (1)]. Infectivity could be maintained through several subcultures. Lesion size was time and dose dependent. A dose of 10^7 amastigotes of L. mexicana or L. braziliensis produced lesions for testing 4 and 6 weeks after infection, respectively.

B. Standardization of Antimony

As per our standard protocol in which mice were inoculated intradermally with 0.1 ml containing 10^7 amastigotes or stationary phase promastigotes at the naired base of the tail, we determined that: (1. neither Pentostam nor Glucantime cured early or late BALB/c infections of L. m. mexicana or L. b. panamensis, (2. Glucantime was somewhat more effective suppressing established infections of L. b. panamensis, and (3. repeated therapy regimes did not improve the efficacy of either drug

against L. mexicana or L. braziliensis infections (Annual Report 1, Figures 6-9). The ED 50 and 90 for suppressing cutaneous or mucocutaneous lesions was one to two logs greater than that required to cure visceral L. donovani infections in BALB/c mice: 461 - 1200 mg/kg/day (mkd) x 5 and 29 or 58 mkd, respectively. (Annual Report 1, Figs. 6-9, Table 1).

Based upon these data, 400 mkd pentavalent antimony x 5, 10, or 15 days was selected as the standard Pentostam dose against which experimental drugs would be tested. The differences both between treatment of early and late L. braziliensis infections, and the greater efficacy of Glucantime v/s Pentostam is not understood at this time. Self-healing has been reported in another experimental L. braziliensis panamensis model (2).

C. WRAIR Screening

1. Aminoquinolines

Once established, these two test systems were used to screen primaquine phosphate and five 8-aminoquinolines identified as potential antileishmanial agents in the hamster primary test system [Annual Report 2, Table 1 (3)]. In our cutaneous and mucocutaneous test systems, none had significant suppressive activity, and none was competitive with Pentostam (Annual Report 2, III.A., Table 7). Three aminoquinolines (WR-211-666, 227-495, and 241-317) were significantly toxic for mice in this test system. Therefore, none of these was recommended for further testing (Annual Report 2, IV. A).

2. Imidazoles

Based upon negative results in our visceral test system (Annual Report 2, III. B.), the imidazole ketoconazole and its acid hydrolysate were not recommended for testing in our cutaneous and mucocutaneous models.

3. Combination Chemotherapy

a. Topical Pentostam

During the second year of this contract the efficacy of topical Pentostam alone or in combination with systemic Pentostam was tested against L. braziliensis infections. Pentostam cream alone had no effect, and there was no potentiation using combination chemotherapy (Annual Report 2, III. C.1, Tables 9 & 10). Lack of enhancement is most likely due to the absence of or inappropriate metabolism of pentavalent antimony in the skin to its active trivalent form (Annual Report 2, IV. C.1). Topical trivalent antimony may have been more useful. If topical antimony or other creams are to be developed, we recommend that in vitro studies using labelled compounds and skin slices, as in topical steroid trials (4), be used to provide more direct evidence for potential efficacy prior to animal screening. More

needs to be understood about the mode of action of Pentostam and its percutaneous absorption by the skin.

b. Pentostam and BCG

A modification of the secondary cutaneous test system was used to test the effect of BCG in combination with Pentostam against L. m. amazonensis infections in BALB/c mice (Annual Report 2, III. C.2). Results showed faster initial resolution of lesions, but no cures were obtained. This is probably due both to the ability of the parasite to use metabolic pathways other than glycolysis, which Pentostam affects (5,6), and to incomplete understanding of the specific mode of action of BCG against New World cutaneous leishmaniasis. Because of promising results in our test system and those of others (7-9), we recommend combination chemotherapy using BCG, Pentostam, and DFMO be further explored (Annual Report 2, IV. C.2).

c. Polyamine Inhibitors

Based upon preliminary data in the visceral model (Annual Report 2, Tables 11 & 12), we are designing experiments to test the efficacy of the polyamine inhibitor DFMO in combination with known and experimental antileishmanial compounds against cutaneous and mucocutaneous infections.

II. Visceral Test System

A. Protocol

Using a modification of the hamster (10) and mouse (11) test systems, we have determined that BALB/c mice inoculated intracardially (IC) with 0.1 ml splenic amastigotes or stationary phase promastigotes produced reliable visceral burdens for screening 7 to 14 days later (Annual Report 1). Intracardial injections allow direct comparison of this test system with the hamster primary screening model.

B. Antimony Standardization

The ED 50 and 95 for Pentostam against L. donovani Sudan strain infections in this test system were determined to be 29 and 58 mkd x 5 days, respectively. Based upon these data, 40 mkd was selected as the standard Pentostam dose against which experimental drugs would be tested in the visceral model.

C. WRAIR Screening

1. Aminoquinolines

Because this test system is rapid (14 days), we used it first to quickly test the activity of five 8-aminoquinolines, primaquine phosphate, and two imidazoles sent to us by WRAIR

(Annual Report 2, Table 1).

The 8-aminoquinolines tested, which showed promise in the primary hamster model, were also active in our secondary BALB/c test system, as indicated by Pentostam Indices of 1.4 to 11.8 (Annual Report 2, Table 7). However, all spleens from these mice were culture positive. No cures were obtained, and none of the Therapeutic Indices for these agents was competitive with Pentostam (TI = 1.20 - 2.00 versus 160). The LD50 of Pentostam was 600 mkd, whereas that for WRAIR aminoquinolines was 10 to 125 mkd (Annual Report 2, IV. A). Based upon these data, none of the WRAIR aminoquinolines was recommended for testing in our cutaneous or mucocutaneous test system (Annual Report 2, IV. A). The toxicity of primaquine phosphate and the 8-aminoquinolines observed was consistent with that known for these compounds, and was probably due to gastrointestinal intolerance and methaemaglobinemia (12).

2. Imidazoles

The visceral model was also used to evaluate the antifungal imidazole ketoconazole and its acid hydrolysate (Annual Report 2, Tables 8). Although these drugs have in vitro activity against species of Trypanosoma and Leishmania (13-14), neither was active in our secondary test system (Annual Report 2, III. B). Ketoconazole disrupts ergosterol synthesis (15), and ergosterol is a major component of leishmania promastigote membranes (16). This imidazole inhibits promastigote sterol biosynthesis in the presence and absence of serum (Berman, J.D., G.G. Holz, Jr. and D.H. Beach, 1983, Leishmania sterol biosynthesis is inhibited by ketoconazole, Abstract 16, 36th Ann. Meeting, Soc. Protozool., Pace Univ., NYC, 20-24 June), but its uptake and specific effect upon intracellular amastigotes was not tested. Within the host, cholesterol is probably preferentially catabolized for biosynthesis of membranes (15). This may account for the inefficacy of ketoconazole in vivo. In humans, near toxic doses for several months must be used to promote healing (17-18). Therefore, neither of these drugs is superior to Pentostam and neither was requested for testing in our cutaneous L. mexicana or L. braziliensis systems. The manufacturers have determined this drug will not be further developed for use in humans (H. Van den Bossche, Pers. Commun.).

3. Combination Chemotherapy

a. Pentostam and BCG

The effect of BCG and Pentostam was not tested in our visceral model . Its efficacy has already been shown by others (7).

b. Polyamine Inhibitors

During the first year of this contract, the polyamine inhibitor DFMO alone was tested in our visceral system and showed no activity (Annual Report 1, Table 2). DFMO alone is active against bloodsteam trypanosomes (19), but its action was potentiated when it was combined with the antitumor drug Bleomycin (20). DFMO alone in our preliminary trials was inactive against L. donovani infections probably because intracellular leishmania divide more slowly than do extracellular trypanosomes. DFMO is preferentially taken up by rapidly dividing cells (19). In a subsequent series of tests, however, 1% DFMO in the drinking water, before or at time of infection, in combination with 3 mkd Bleomycin, suppressed infections 91% or 87%, respectively (Annual Report 2, Tables 11 & 12). DFMO and Bleomycin did not cure all mice, but the treatment was competitive with Pentostam, as measured by cultures and impression smears. Liver burdens were suppressed 46% even when treatment was begun 3 days after infection.

This is the first time in more than 30 years that any compound has been competitive with antimonials. DFMO is non-toxic and specific for an essential pathway in leishmania (Annual Report 2, Fig. 4, Tables 13 & 14). Its prophylactic effect and ease of delivery suggest this agent be given priority for further trials in combination with known, active compounds eg. Pentostam, Pentamidine, and with the most promising new, experimental compounds eg. WR 6026, allopurinol riboside, Lampit, Radanil.

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